EFFECT OF pH AND IONIC STRENGTH ON MEMBRANE FLUX IN THE SEPARATION OF *Escherichia coli* BY USING CROSS-FLOW MICROFILTRATION

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ABSTRACT

Recently, cross-flow microfiltration has been used to separate cells in continuous fermentation processes. The main objective of this study is to investigate the effect of pH and ionic strength on membrane flux during the separation of *Escherichia coli*. In order to achieve the objective of the study, hollow fibre membrane with molecular pore size of 0.2µm and surface area of 110 cm^2 was used as the filter media. The experiment was carried out at a constant transmembrane pressure of 0.8 bar by varying five different pH values, ranging from pH 4.5 to 8.5 and five different concentration of salt, ranging from 0.1M to 0.5M. From this study, it is found that pH 6.5 with low concentration of salt was the best condition for the separation of *E.coli*. Increasing in pH increased the permeate flux, while high concentration of salt decreased the permeate flux. As a conclusion, the membrane used in this experiment can be used in the fermentation of *E.coli* for cell recycling because pH 6.5 is the optimum condition for fermentation of *E.coli* in its fermentation broth.

ABSTRAK

Sejak kebelakangan ini, penapis mikro telah digunakan untuk memisahkan sel di dalam proses penapaian secara berterusan. Tujuan utama kajian ini dijalankan adalah untuk mengkaji kesan pH dan kekuatan ionik ke atas arus membran di dalam pemisahan *Escherichia coli.* Dalam mencapai objektif kajian, membran jenis fiber berongga dengan saiz liang molekul 0.2µm dan luas permukaan 110 cm^2 telah digunakan sebagai media penapis. Eksperimen ini telah dijalankan pada tekanan yang tetap, iaitu 0.8 bar dengan mempelbagaikan lima nilai pH yang berbeza, iaitu di dalam julat 4.5 hingga 8.5 dan lima kepekatan garam yang berbeza, iaitu di dalam julat 0.1 molar hingga 0.5 molar. Kajian ini menunjukkan bahawa pemisahan *E.coli* yang terbaik berlaku pada pH 6.5 dengan kepekatan garam yang rendah. Arus resapan meningkat dengan peningkatan pH, manakala kepekatan garam yang tinggi mengurangkan arus resapan. Kesimpulannya, membran yang telah digunakan di dalam eksperimen ini boleh digunakan di dalam penapaian *E.coli* untuk sel dikitar semula kerana pH 6.5 adalah keadaan optimum untuk menapai *E.coli* di dalam medium penapaiannya.

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LIST OF SYMBOLS/ABBREVIATIONS

E. coli	-	*Escherichia coli species*
µm	-	micrometer
cm	-	centimeter
LPG	-	liquefied petroleum gas
M	-	molar
NaCl	-	sodium chloride
TCA	-	tricarboxylic acid
CO_2	-	carbon dioxide
LB	-	Luria Bertani
PBS	-	polybutyrate succinate
NaOH	-	sodium hydroxide
PHA	-	polyhydroxyalkanoate
°C	-	degree Celcius
DNA	-	deoxyribonucleic acid
pH	-	potential for hydrogen ion concentration
MF	-	microfiltration
rpm	-	revolution per minute
TMP/ΔP	-	transmembrane pressure
UF	-	ultrafiltration
NF	-	nanofiltration
mL	-	mililitres
min	-	minute
pI	-	isoelectric point

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Succinic acid is a dicarboxylic acid having the molecular formula of $C_4H_6O_4$. It has been produced by microbial fermentation for the use in agricultural, food and pharmaceuticals industries. Currently, most of commercially available succinic acid is produced by chemical processes, in which liquefied petroleum gas (LPG) or petroleum oil is used as a starting material. However, the assessment of raw material cost and the estimation of the potential market size clearly indicate that the current petroleum-based succinic acid process will be replaced by the fermentative succinic acid production system in the foreseeable future (Song and Lee, 2006).

Many different microorganisms have been screened and studied for for succinic acid production from various carbon sources. *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes* and *Mannheimia succiniciproducens* MBEL55E are among the bacterias found to be used in the fermentation of succinic acid due to their ability to produce a relatively large amount of succinic acid (Song and Lee, 2006). However, there

has been much effort in developing recombinant *Escherichia coli* strains which are capable of enhanced succinic acid production whether under aerobic or anaerobic conditions.

A typical process for the production of a bioproduct like succinic acid by microbial fermentation consists of seed cultivation, fermentation, product recovery, concentration and purification (Song and Lee, 2006). The separation of cells is the first separation step after fermentation either to recover extracellular products, to concentrate and wash cells before product recovery, or for cell recycle. Usually, the most common methods for this separation are centrifugation and plate and frame filters. However, these methods have its own limitations. The only other technique which has been suggested for cell separation is sedimentation. Nevertheless, this technique requires long residence times and always requires another separation step (Warren *et al.*, 1991).

Recently, cross-flow microfiltration has been used to separate cells in continuous fermentation processes. A successful succinic acid recovery approach in continuous fermentation is in a cell-recycled reactor where the cells are separated by a filtration unit and returned to the fermenter while the product is removed in the permeate (Li *et al.*, 2006).

The use of synthetic dead-end membrane filters has now become standard in any biological laboratory. However, for industrial cell separations, where a continuous high throughput stream is required, this design is inadequate because of the huge resistance created by cell build-up on the upstream side of the membrane. Instead, a cross-flow or tangential flow system, in which the membrane surface is parallel to the inlet flow has been suggested. This allows much of the cellular fouling on the membrane surface to be eliminated by the cells being swept away by the tangential flow, so a steady state exists where the rate of deposition due to the filtrate flow is balanced by the rate of removal by

the cross-flow. The filter can then be operated for considerably longer time periods and with higher fluxes than dead-ended filtration (Warren *et al.*, 1991).

The efficiency of cross-flow microfiltration is primarily a function of the operating parameters, and is measured by the filtrate flow rate (flux) and its quality. Cross-flow velocity, transmembrane pressure, temperature, pore size of the membrane and concentration of suspended solids in the feed were reported to affect the performance of cross-flow microfiltration (Al-Malack *et al.*, 2004).

In this study, cross-flow microfiltration system with hollow fibre membrane as the filter media was implemented to separate *E.coli* cells from its fermentation broth. *E.coli* is essential in the production of succinic acid. In industrial cell separations, hollow fibre membrane is located in the fermenter. It is functioning to retain the cells in the fermenter for cell-recycling, while the product which is succinic acid is removed in the permeate. Cell-recycling can reduce the production cost of succinic acid. Hollow fibre membrane is used in this filtration system instead of other types of membrane such as flat sheet due to its minimum-required space. The production cost of succinic acid can be reduced without spending much money on larger equipments which also requires larger space. This study investigates the effect of pH and ionic strength on the membrane flux to determine the optimum condition for the separation of *E.coli*. The most suitable pH and ionic strength in the separation of *E.coli* will enhance high flux and high rejection of cells.

1.2 Problem Statement

Recently, increasing interest has been generated in the separation of cells by cross-flow filtration. This technique is usually used as the first separation step after fermentation either to recover extracellular products, to concentrate and/or wash cells before product recovery, or for cell recycle. Presently, the most common methods for this separation are centrifugation and plate and frame filters. However, centrifuges (i) have a high complexity and cost, (ii) often leave turbid supernatants, (iii) require high gravitational forces and (iv) can create aerosols; while plate and frame filters (i) are labour intensive, (ii) have fluxes which decrease with time, (iii) waste significant amounts of product, (iv) require filtering aids and (v) have problems with hygiene. The only other technique which has been suggested for cell separation is sedimentation. Although inexpensive, this technique requires long residence times and, because it produces imperfect separations, sedimentation usually requires another cell separation step (Warren *et al.*, 1991).

Cross-flow microfiltration can avoid those limitations. Nevertheless, membrane-based separation often faced with membrane fouling. The long term performance of membrane units at high cell densities is always affected by the fouling of filtration membranes, which require extensive cleaning protocols (Li *et al.*, 2006).

1.3 Rationale and Significance

To avoid limitations mentioned in the previous sub-chapter, cross-flow microfiltration technique was chosen as the separation method because it allows continuous and complete separations which are not dependent upon the density

difference or particle size and can be performed in a contained system which is both sterile and aerosol-free (Warren *et al.*, 1991). In cross-flow or tangential microfiltration, the particles deposited on the filter medium are swept away by the cross-flow velocity action, which produces shear and lift forces on the particles as they become attached to the filter medium (Al-Malack *et al.*, 2004). Membrane with higher flux and rejection of cells is preferred to separate *E.coli* from its fermentation broth.

Membrane fouling, which results in loss of productivity, is one of the major operational concerns of membrane processes (Tansel *et al.*, 2000). Further study on membrane fouling has been done to increase the flux, hence reduce fouling and increase the life-span of the membrane. Extensive cleaning protocols are essential in order to increase the life-span of the membrane.

1.4 Objective

This main objective of this study was to investigate the effect of pH and ionic strength on membrane flux during the separation of *E.coli*.

1.5 Scope of Study

In order to achieve the objective of the study, the following scopes have been identified:

1) The value of pH was varied between pH 4.5 to pH 8.5.
2) The concentration of sodium chloride (NaCl) was varied from 0.1M to 0.5M.
3) Hollow fibre membrane with molecular pore size of 0.2 µm and surface area of 110 cm^2 were used.
4) Constant transmembrane pressure of 0.8 bar was implemented.

CHAPTER 2

LITERATURE REVIEW

2.1 Succinic Acid

Succinic acid, also known as amber acid or butanedioic acid, is a dicarboxylic acid having the molecular formula of $C_4H_6O_4$. After its first purification of succinic acid from amber by Georgius Agricola in 1546, it has been produced by microbial fermentation for the use in agricultural, food and pharmaceutical industries (Song and Lee, 2006). Traditionally, succinic acid is produced by chemical synthesis from petroleum feedstocks which are not renewable. Succinic acid is an intermediate metabolite in the tricarboxylic acid (TCA) cycle, and can be produced by some obligate or facultative anaerobes. It is considered one of the most possible commercial products obtained from alternative feedstocks. Therefore, production of succinic acid from renewable substrates has been investigated in recent years for sustainable development (Wu *et al.*, 2009).

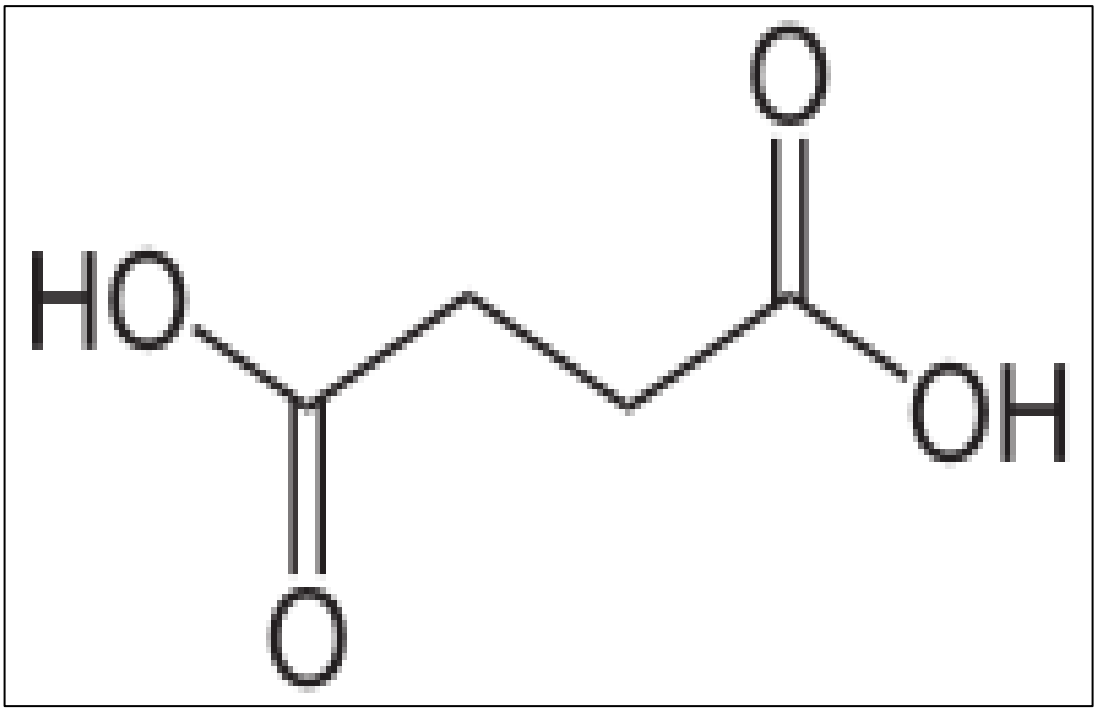

Figure 2.1 Molecular structure of succinic acid

2.1.1 Succinic Acid Production

Succinic acid is currently chemically produced by hydrolyzing petroleum products, which is associated with certain environmental hazards leading scientists to develop biological processes for its continuous production. This is because it is a common intermediate in the metabolic pathway of several anaerobic microorganisms. Efforts are being made worldwide to develop low-cost fermentation processes using renewable resources such as agricultural, dairy, and industrial waste products, so as to replace current processes using petroleum as a feedstock (Agarwal *et al*., 2007).

As the importance of succinic acid for use as a biodegradable polymer has increased, the biological production by fermentation has been focused on as the alternative to the petrochemical-based process (Huh *et al.*, 2006). Many different

microorganisms have been screened and studied for succinic acid production from various carbon sources. Among them, *Anaerobiospirillum succiniciproducens* and *Actinobacillus succinogenes* have been most intensively studied due to their ability to produce a relatively large amount of succinic acid. More recently, a new succinic acid producing bacterium *Mannheimia succiniciproducens* MBEL55E was isolated from bovine rumen. Also, there has been much effort in developing recombinant *Escherichia coli* strains which are capable of enhanced succinic acid production under aerobic and anaerobic conditions (Song and Lee, 2006). Table 2.1 shows various kinds of microorganism that can produce succinic acid.

Table 2.1 Succinic acid production from various microorganisms

Microorganism	Description	Reference
Actinobacillus succinogenes	Fermentative production of succinic acid from straw hydrolysate.	Zheng *et al.*, 2009
Mannheimia succiniciproducens	Produces succinic acid as a major product, acetic and formic acids as the second major ones from various carbon sources under 100% CO_2 condition at pH of 6.0 to 7.5.	Huh *et al*., 2006
Anaerobiospirillum succiniciproducens	Produces succinic and acetic acids as major fermentation products and ethanol and lactic acid as minor ones under strictly anaerobic condition.	Lee *et al*., 1999
Recombinant Escherichia coli	Ferments glucose to ethanol, formic, acetic and lactic acids with only detectable amounts of succinic acid under anaerobic condition.	Song and Lee, 2006
Bacteroides fragilis	Produces a polysaccharide capsule high in succinic acid.	Isar *et al*., 2006

2.1.2 Application of Succinic Acid

Succinic acid can be used as a precursor of many industrially important chemicals including adipic acid, 1,4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts and gamma-butyrolactone. Furthermore, the increasing demand for succinic acid is expected as its use is extended to the synthesis of biodegradable polymers such as polybutyrate succinate (PBS) and polyamides, and various green solvents (Song and Lee, 2006). Figure 2.2 shows various chemicals and products that can be synthesized from succinic acid.

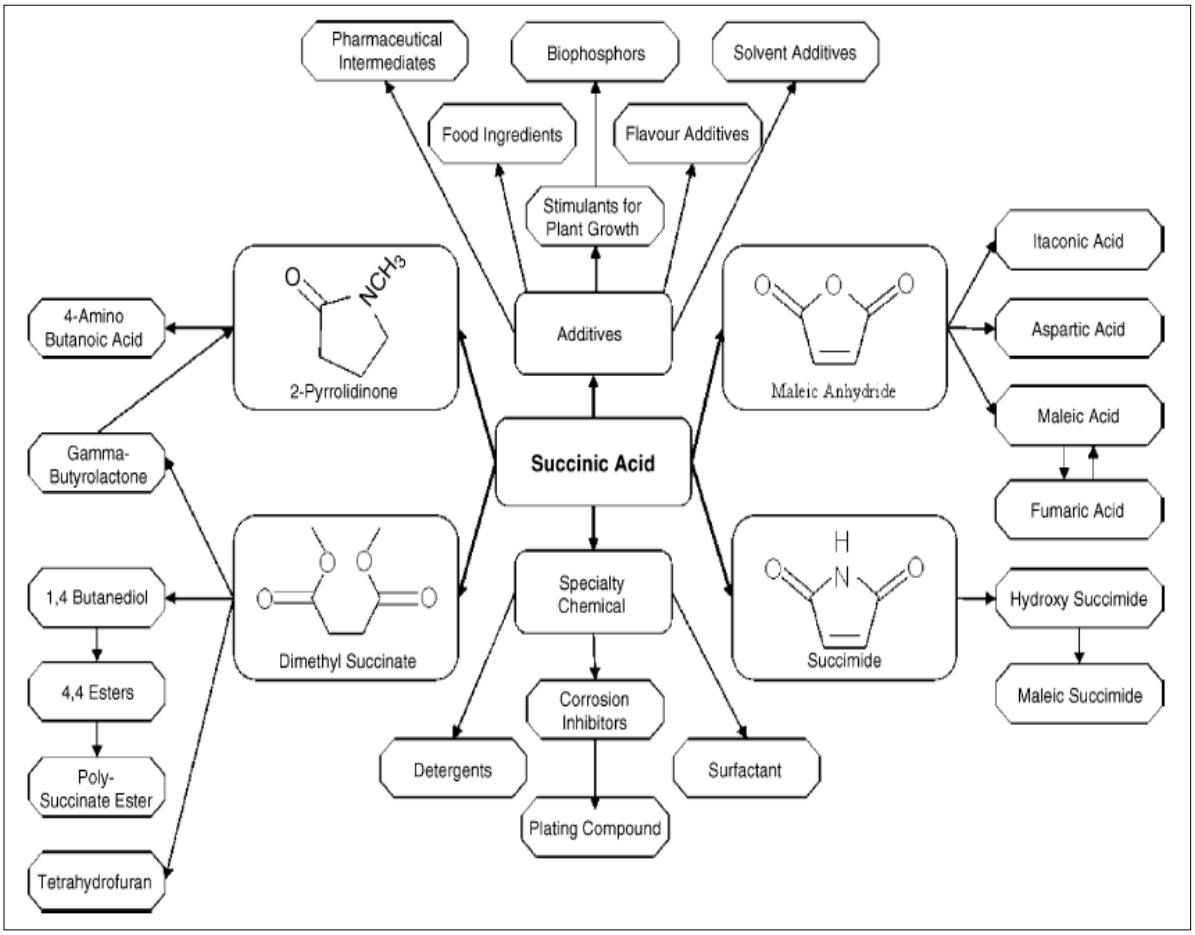

Figure 2.2 Various chemicals and products that can be synthesized from succinic acid

2.2 *Escherichia coli sp.*

Escherichia coli was first described and isolated by Theodore Escherich in1885. *E.coli* strains K-12 and B are apparently both derived from normal commensals of the human gut, and their many derivatives have been in laboratory since 1922 and before 1918, respectively (Jeong, *et al.*, 2009). *E.coli* is a gram-negative, facultative anaerobic and non-sporulating cell. It is typically rod-shaped and is about 2 micrometres (µm) long and 0.5 µm in diameter. It has a cell volume of 0.6 to 0.7 μm^3 (Kubitschek, 1990). It can live on a variety of substrates. *E.coli* uses mixed-acid fermentation in anaerobic conditions to produce lactate, succinate, ethanol, acetate and carbon dioxide.

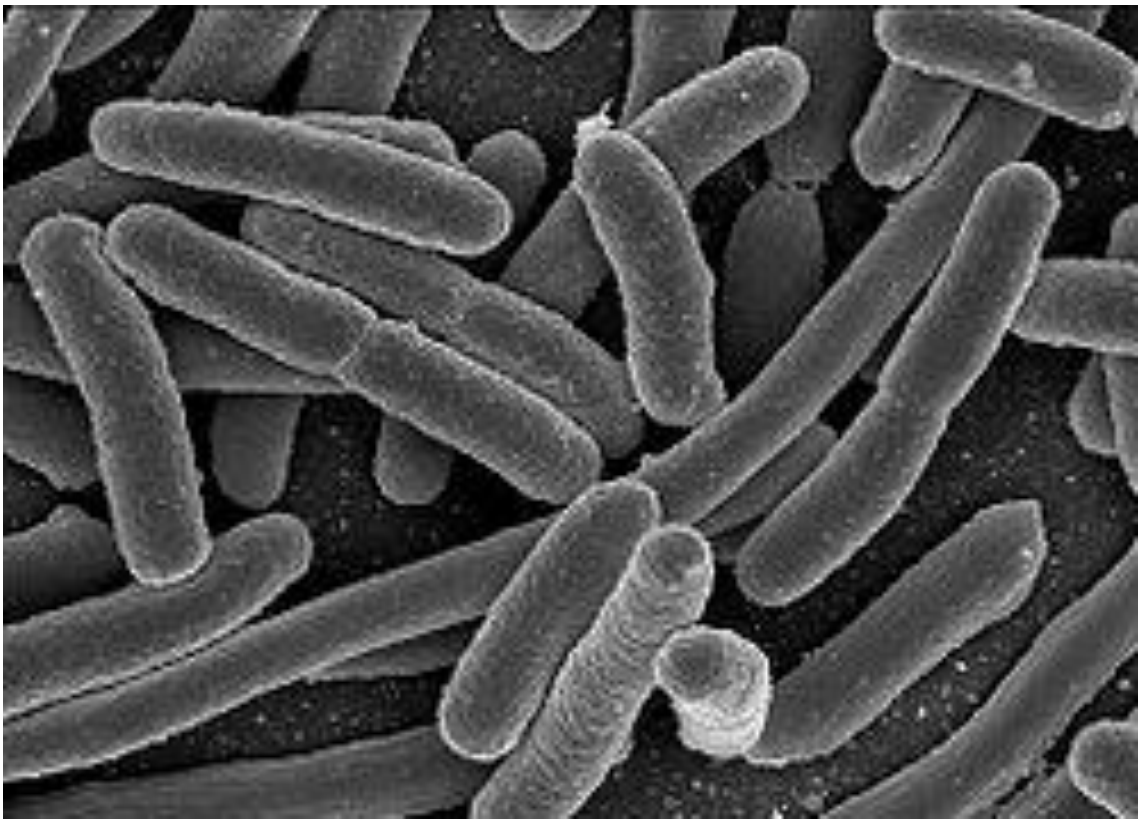

Figure 2.3 *Escherichia coli sp.*

2.2.1 Industrial Application of *Escherichia coli*

Current commercial products obtained from *E.coli* cultures include mainly recombinant proteins from prokaryotic and eukaryotic sources which are considered to be low-volume-high-value products. In addition, recent advancements in metabolic engineering made it possible to use *E.coli* as a platform to produce high-volume-low-value-products such as polyhydroxybutyrate, succinic acid, octanoic acid, aromatic compounds, ethanol, acetone and styrene oxide (Shiloach and Fass, 2005). Table 2.2 shows some applications of *E.coli* in production of bioproducts.

Table 2.2 Application of *E.coli* in production of bioproducts

Application	Description	Reference
Production of Penicillin Acylase (PAC)	An important industrial enzyme for the production of many β-lactam antibiotics.	Chou *et al.*, 1999
Production of L-aspartic acid	Immobilization of *Escherichia coli* cells using polyethyleneimine-coated porous support particles for L-aspartic acid production.	Huang *et al.*, 2009
Production of L(-)-Carnitine	L(-)-Carnitine production with immobilized *Escherichia coli* cells in continuous reactors.	Obon *et al.*, 1997
Production of ethanol from xylose	*E. coli* FBR5 is developed to produce ethanol in high yields from corn fibre hydrolysate and other agricultural residues.	Quresh *et al.*, 2006
Industrial production of polyhydroxyalkanoates	*E. coli* is used due to higher growth rates and PHA production levels, nutrient limitation is not required, and easier PHA recovery due to cell fragility and larger granule size	Wegen *et al.*, 1998

2.2.2 Production of Succinic Acid from *Escherichia coli*

A number of metabolic engineering strategies have been developed to enhance succinic acid production by *E.coli*. The approaches can be broadly categorized to the inactivation of enzymes participating in the reactions which compete with succinic acid pathways, the amplification of enzymes involved in succinic acid pathways, and the introduction of heterologous enzymes catalyzing reactions towards increased succinic acid formation (Song and Lee, 2006).

2.3 Cultivation of *Escherichia coli*

Microbiological cultures utilize petri dishes of differing sizes that have a thin layer of agar based growth medium in them. Once the growth medium in the petri dish is inoculated with the desired bacteria, the plates are incubated in an oven usually set at 37 degrees Celsius (°C). Another method of bacterial culture is liquid culture, in which case desired bacteria are suspended in liquid broth, a nutrient medium. These are ideal for preparation of an antimicrobial assay. The experimenter would inoculate liquid broth with bacteria and let it grow overnight in a shaker for uniform growth, then take aliquots of the sample to test for the antimicrobial activity of a specific drug or protein.

2.3.1 Aseptic Technique

Aseptic techniques must be used to reduce the likelihood of bacterial contamination. This usually involves disinfection of working areas, minimizing possible access by bacteria from the air to exposed media, and use of flames to kill bacteria

which might enter vessels as they are opened. *E.coli* may be introduced to the media (inoculated) by various means. Usually, *E.coli* from a drop in a heat-sterilized loop is spread on the surface of agar. A similar technique is used with broth cultures.

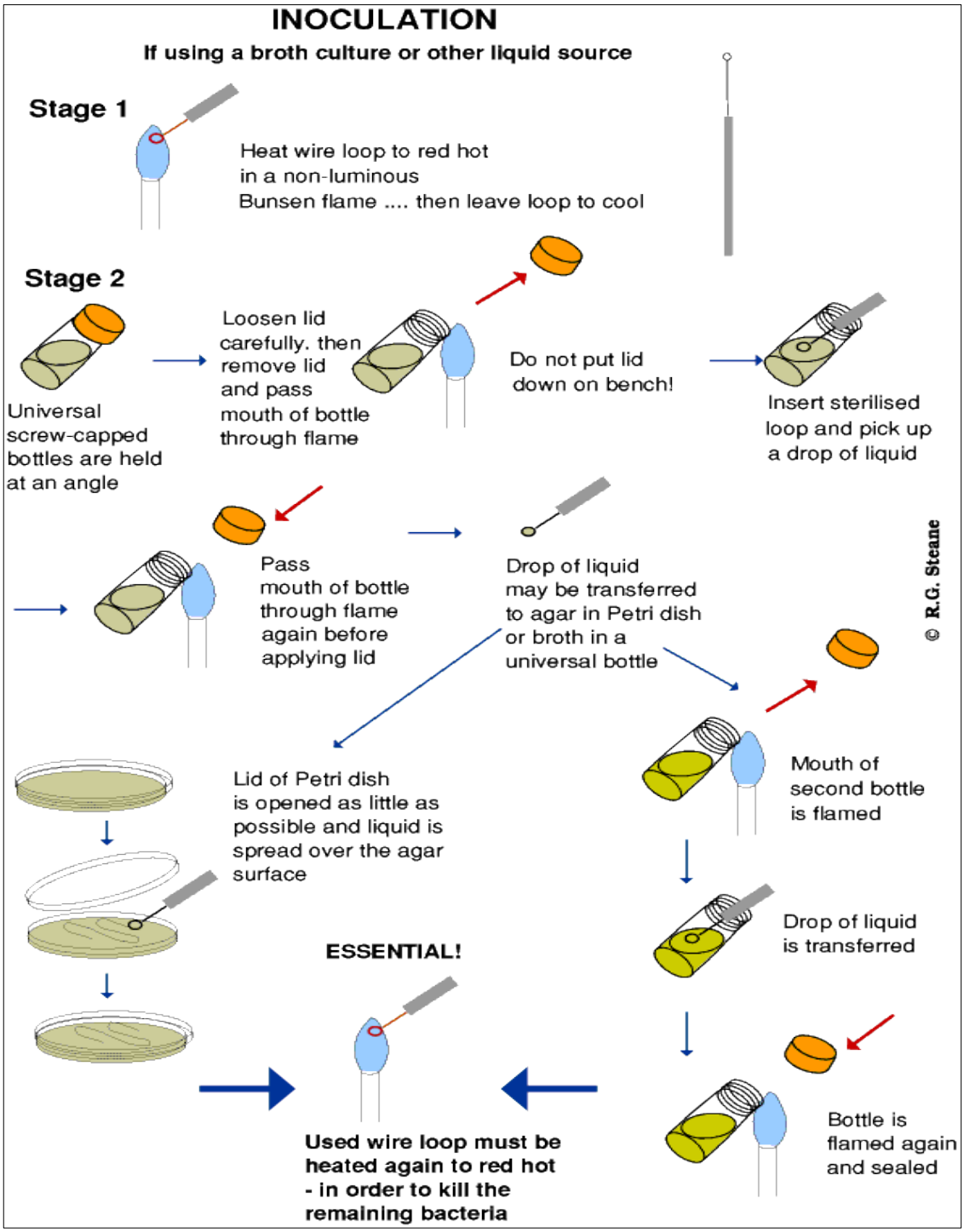

Figure 2.4 Inoculation techniques for *Escherichia coli*

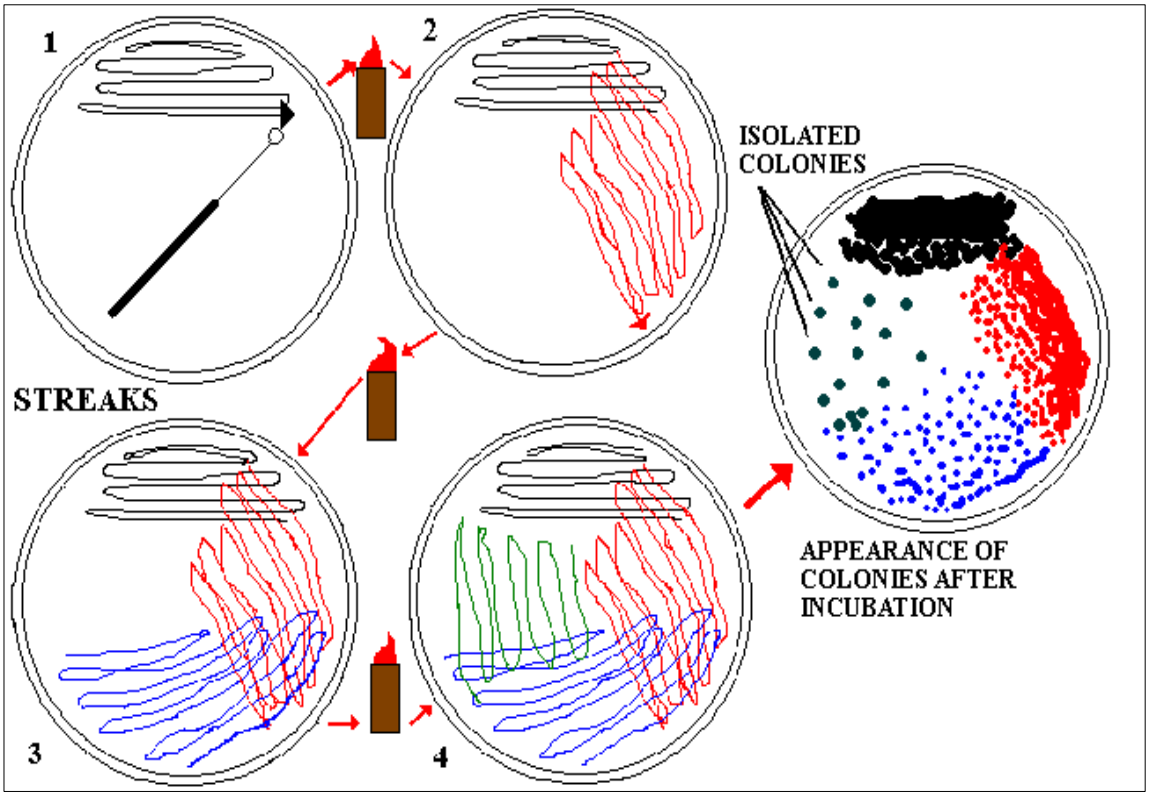

Figure 2.5 Streak plate methods

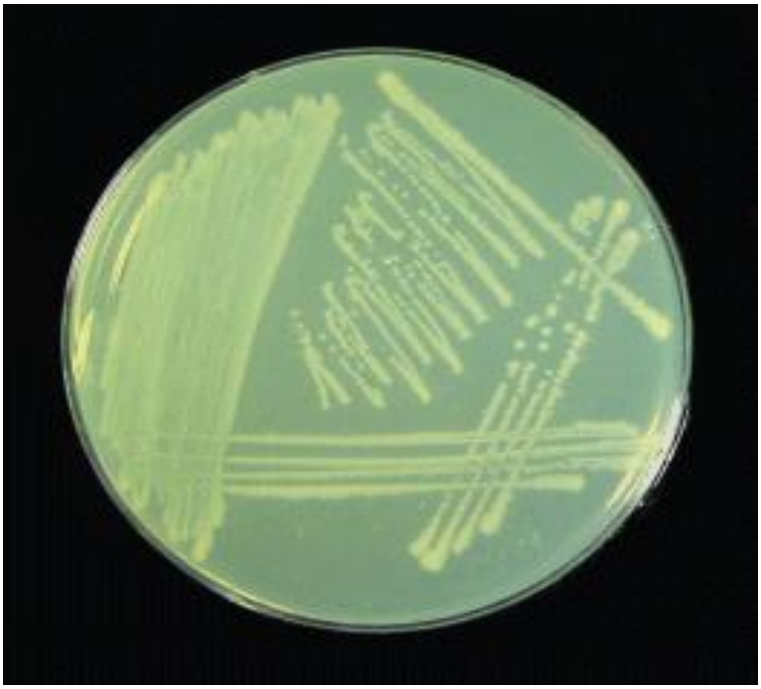

Figure 2.6 *E.coli* on agar plate after incubation

2.3.2 Luria Bertani Broth

The widely used rich medium called Luria Bertani (LB) broth is popular with bacteriologists because it permits fast growth and good growth yields for many species. LB media formulations have been an industry standard for the cultivation of *E.coli* as far back as the 1950s. These media have been widely used in molecular microbiology applications for the preparation of plasmid DNA and recombinant proteins. It continues to be one of the most common media used for maintaining and cultivating recombinant strains of *E.coli*. (Sezonov *et al.*, 2007).

There are several common formulations of LB. Although they are different, they generally share a somewhat similar composition of ingredients used to promote growth, including peptides and casein peptones, vitamins (including B vitamins), trace elements (nitrogen, sulphur, magnesium) and minerals. Peptides and peptones are provided by tryptone. Vitamins and certain trace elements are provided by yeast extract. Sodium ions for transport and osmotic balance are provided by sodium chloride. Bacto-tryptone is used to provide essential amino acids to the growing bacteria, while the bacto-yeast extract is used to provide a plethora of organic compounds helpful for bacterial growth. The tryptone used is a pancreatic digest of casein from cow's milk, and the yeast extract used is an autodigest of *Saccharomyces cerevisiae* (Sezonov *et al.*, 2007).